35. (New) A method for isolating, purifying, or identifying a tumor suppressor gene associated with hepaticellular carcinoma in a patient comprising:

a) constructing a cosmid library from a YAC clone containing a genomic DNA of interest;

b) selecting cosmid clones of interest by colony hybridization with labeled human genomic DNA as a probe;

c) performing an exon amplification reaction using DNA from the selected cosmid clones and resulting in reverse transcribed sequences;

d) hybridizing the reverse transcribed sequences with a human cDNA library; and

e) selecting hybridized cDNA clones.

REMARKS

Applicants now respond to the Notice to File Missing Parts, mailed on June 1, 2001 by paying the basic filing fee based on the claim amendments and additions in this Preliminary Amendment.

Applicants have canceled claims 2-17 and 21-27 and added new claims 28-35. Thus, claims 1, 18-20, and 28-35 are pending in the instant application. In an effort to reduce the required filing fee, Applicants have retained the subject matter of the non-allowed claims in the parent 09/055,353 patent application ('353 application) in new claims 28-35. In condensing these claims, Applicants reserve the right to prosecute the subject matter of all of the non-allowed claims in the '353 application.

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Applicants have amended the specification to reflect changes that were introduced via a Preliminary Amendment filed on October 23, 1998 in the '353 application. These changes correct typographical and grammatical errors present in the original specification. Thus, these amendments to the specification do not constitute new matter.

If there are any additional fees due in connection with the filing of this Preliminary Amendment, please charge the fee to our Deposit Account No. 06-0916.

Respectfully submitted,

FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: September 4, 2001

By: Rebecca M. McNeill
for Kenneth J. Meyers Reg. No. 43,796
Reg. No. 25,146

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Application Number: 09/819,007
Filing Date: March 28, 2001
Attorney Docket Number: 3495.0168-01

APPENDIX TO AMENDMENT OF SEPTEMBER 4, 2001

Amendments to the Specification

Please amend the specification as follows:

On page 1, after the title, insert the following:

--CROSS-REFERENCE TO RELATED APPLICATION

This application hereby claims the benefit under 35 U.S.C. §119(e) of United States application S.N. 09/055,353, filed April 6, 1998 which claims the benefit of provisional application S.N. 60/043,437, filed April 7, 1997. The entire disclosure of this application is relied upon and incorporated by reference herein.--

On page 2, replace the paragraph beginning on line 6 with the following new paragraph:

Generally, the development of human cancer results from clonal expansion of genetically modified cells that acquired selective growth advantage through accumulated alterations of [ptoto] photo-oncogenes and tumor suppressor genes (Weinberg, 1991). Somatic inactivation of tumor suppressor genes is usually achieved by intragenic mutations in one allele of the gene and by the loss of a chromosomal region spanning the second allele.

On page 2, replace the last paragraph beginning on line 31 continuing to page 3 with the following new paragraph:

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Chromosomal analysis using polymorphic DNA markers that distinguish different alleles has revealed loss of hereozygosity (LOH) of specific chromosomal regions in various types of cancers and the mapping of regions with a high frequency of LOH has been critical for identifying negative regulators of tumor growth (Call et al., 1990; Fearon et al., 1990; Friend et al., 1986). The recent development of [microstaellite] microsatellite polymorphic markers has allowed positional cloning of several tumor suppressor genes such as the BRCA1, BRCA2 and DPC4 genes (Hahn et al., 1996; Miki et al., 1994; Wooster et al., 1995).

On page 3, replace the first paragraph beginning at line 4 with the following new paragraph:

Previous studies, mainly relying upon either restriction fragment length polymorphism (RFLP) markers or [microsatellites] microsatellite markers restricted to specific chromosome arms, have defined a number of chromosomal regions of LOH in liver cancer. One of the most frequent allelic deletions in HCC has been found at chromosome 17p where the tumor suppressor gene p53 is located (Fujimori et al., 1991; Murakami et al., 1991; Slagle et al., 1991). The frequency of p53 mutations varies largely among HCC samples, depending on the geographic location in the world, and a hot spot mutation at codon 249 was observed in HCCs from regions with high levels of dietary aflatoxins and high prevalence of HBV infection (Bressac et al., 1991; Buetow et al., 1992; Hsu et al., 1991). Regional deletions spanning the RB locus on chromosome 13q have also been described, but in this

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case, a low mutation rate was found in the remaining allele (Murakami et al., 1991; Wang and Rogler., 1988; Zhang et al., 1994). The most frequent chromosome arm deletion is observed in 13q(13q12-q32) which harbors the RB and BRCA2 tumor suppressor genes (Friend et al., 1986; Wooster et al., 1995; Zhang et al., 1994). Other frequent LOH was reported on chromosome arms 1p, 4q, 5q, 6q, 8p, 10q, 11p, 16p, 16q and 22q (Buetow et al., 989; De Souza et al., 1995; Emi et al., 1992; Fujimori et al., 1991; Takahashi et al., 1993; Tsuda et al., 1990; Wang and Rogler, 1988; Yeh et al., 1994). Candidate tumor suppressor genes in these regions include the mannose 6-phosphate/insulin-like growth factor II receptor gene (M6P/IGF2R) on 6q26-q27 (De Souza et al., 1995), the PDGF-receptor beta-like tumor suppressor gene (PRLTS) on 8p21-p22 (Fujiwara et al., 1995) and the E-Cadherin gene on 16q22 (Slagle et al., 1993).

On page 3, please replace the last paragraph beginning at line 27 with the following new paragraph:

Yeh et al. (1994) have performed a genetic analysis of HCC cell lines and 30 primary HCC Tissues. Using 8 Polymorphic DNA markers for RFLP experiments and also [microsatellites] <u>microsatellites</u> markers spanning 12 loci in chromosome 1p, these authors have shown that main chromosomal abnormalities seemed to cluster at the distal part of chromosome 1p, with a common region mapped to 1p35-36, which is also the region with frequent loss of

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heterozygosity in neuroblastoma and colorectal as well as breast cancers.

On page 4, replace the second paragraph beginning at line 7 with the following new paragraph:

Fujimori et al. (1991) have realized an allelotype study of HCC by examining LOH with 44 RFLP markers in 46 cases of HCC. The markers used by Fujimori et al; represented all chromosomal arms [excepted] except 5p, 8p, 9p, 18p and acrocentric chromosomes. Each chromosomal arm was thus mapped with only a single or two polymorphic RFLP markers. These authors have observed that a significant percentage of LOH occurred for chromosome arms 5q (4 deletions in informative cases [[44% LOH]] (44% LOH)), 10q (6 deletions in 24 informative cases [[25% LOH]] (25% LOH)), 11p (5 deletions in 13 informative cases [[46% LOH]] (46% LOH)), 16q (12 deletions in 33 informative cases [[36% LOH]] (36% LOH)), and 17p (5 deletions in 11 informative cases [[45% LOH]] (45% LOH)).

On page 4, replace the third paragraph beginning at line 16 with the following new paragraph:

Buetow et al., (Buetow et al., 1989) reported LOH at the albumin gene locus (4q11-q12) in all of five informative HCCs, indicating that a tumor suppressor gene might lie in this region. The [inventor's] inventors' data suggest that alterations in two additional loci on chromosome 4q may play a role in liver carcinogenesis. Because chromosome 4q contains genes encoding

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growth factors or genes expressed predominantly in the liver such as albumin, alcohol dehydrogenase (ADH3), fibrinogen and UDP-glucuronyl-transferase, the deletion of this region might profoundly alter cell growth conditions and hepatocyte functions.

Page 4, replace the fourth paragraph beginning at line 24 with the following new paragraph:

Buetow et al. (1989) have studied the LOH in 12 human primary liver tumors that have been tested against a panel of RFLP markers. These authors have typed tumor and non tumor tissue for 11 RFLP markers spanning from 4q11-q13 to 4q32 chromosome 4 regions. In addition, Buetow et al. tested at least one RFLP marker on nine other chromosomes (1 2 6, 7, 9, 11, 13, 14 and 17) for allelic loss. The results showed that seven [on] <u>of</u> nine tumors constitutionally heterozygous for chromosome 4q markers (six 4q RFLP markers were used by Buetow et al.) showed allele loss in tumor tissue. Six of the seven [sample] <u>samples</u> were jointly informative for both 4p and 4q markers (six 4p RFLP markers used). Among the other chromosomes informative for allele loss, one tumor showed changes in 13q. No other changes were observed in RFLP markers located on the eight other chromosomes tested. These authors concluded that controlling locus involved in the pathogenesis of HCC might be in the vicinity of 4q32.

On page 5, replace the first paragraph beginning at line 1 with the following new paragraph:

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Emi et al. (1992) observed a frequent LOH for different loci on chromosome 8p in tumor tissues derived from HCC, colorectal cancer and lung cancer. More particularly, Emi et al. studied LOH in 120 HCC (46 of which had previously [already] been allelotyped by Fujimori et al. in 1991) tissues with five polymorphic markers along the short arm of chromosome 8 and defined commonly deleted regions within the same chromosomal interval, 8p23.1 to 8p21.3, suggesting that one or more tumor suppressor genes for HCC, and also for colorectal cancer, might be present in said region. The region of interest was mapped by Emi et al. using only three RFLP polymorphic DNA markers, respectively D8S238, MSR and D8S220. These authors concluded that a putative tumor suppressor gene might exist on 8p.

Page 5, please replace the third paragraph beginning at line 20 with the following new paragraph:

Boige et al., in 1996, [have] studied the allelic deletions in HCC, using 275 highly polymorphic [microsatellites] microsatellite genetic markers spanning all non acrocentric chromosome arms in a group of 48 HCC. They observed that nine chromosome arms were deleted in more than 30% in 1p, 1q, 4q, 6q, 8p, 9p, 16p, 16q and 17p, the most frequent chromosome arm deletion being observed for 8p.

On page 5, replace paragraph 4, beginning at line 26, continuing to page 6 beginning at line 1, with the following new paragraph:

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The scientific works described hereinbefore have allowed a primary but very coarse localization of the different genetic alterations occurring on the different chromosome arms present in the HCC tissue samples, due either to the weak number of genetic markers used [and] or to the weak number of patients' tissue samples studied. The weak number of patients' tissue sample used in these studies did not provide conclusive or statistically significant data as to the [frequence] frequency of a genetic alteration on a given chromosome arm of chromosome region. The poor precision with which the altered [locuses] loci were identified, and consequently the great size of the chromosome DNA fragments of interest that are bordered by polymorphic DNA markers used in these studies, did not allow [the] one of ordinary skill in the art to design and/or determine the suitable technical means useful to design accurate diagnostic tools for HCC, because none of the DNA fragments was shown in the prior art to be sufficiently relevant and to be considered as carrying the casual information for operating a precise correlation with the disease. Consequently, they did not permit [the] one of ordinary skill in the art to clone specific DNA fragments from the chromosomes of healthy tissues, that were observed to be frequently altered during the occurrence of HCC.

On page 6, replace the second paragraph, beginning at line 9, with the following new paragraph:

Primary liver tumors, like other solid tumors in humans, most likely arise through a cascade of genetic events involving

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oncogenes and tumor suppressor genes that results in decreasing stability of the genome and ultimately leads to the malignant phenotype. The methods used according to the invention, that [allowed] allow an accurate and complete scan of 120 hepatocellular carcinoma genomes for allelic imbalance, are of decisive value for locating candidate genes implicated in liver cancer development.

On page 6, replace the last paragraph beginning at line 32 continuing to page 7, beginning at line 1, with the following new paragraph:

The inventors have also found frequent LOH with markers depicted in Tables 2 and 4, therefore allowing [the] one of skill in the art to determine the presence and characteristics of HCC-associated tumor suppressor genes existing on the different identified loci of interest, particularly on 8p21-8p23, 1p35-p36, 16q23-q24, 14q32, 4q and 6q.

On page 7, replace paragraph 2 beginning at line 9, with the following new paragraph:

The present inventors have now used a very dense panel of polymorphic microsatellite markers in order to precisely localize the different [chromosomes] chromosome regions being altered in HCC [patients] patients' [cancer] liver tissue samples. The inventors have therefore performed a systematic screening of 120 HCC samples using 256 highly polymorphic microsatellite markers evenly distributed throughout non-acrocentric human autosomes.

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This is the first, large scale analysis of genomic alterations in human HCC using microsatellite markers. The data allow precise estimation of the relative frequency and accurate positioning of each chromosomal change.

On page 7, replace paragraph 4 beginning at line 20 with the following new paragraph:

Because the inventors have now precisely identified the very small chromosomal regions that are subject to frequent genetic alterations, the DNA markers embraced by the present invention cover all the publicly available tools spanning these specific chromosomal loci of [interrest] interest, namely, microsatellites DNA markers, namely:

1) Microsatellite DNA markers;

2) RFLP markers (usually constituted by specific oliqonucleotide probes);

3) VNTR markers (Variable Number of Tandem Repeats), also named [<<ministallites>>] <<minisatellites>> that are sequences of the <<Alu>> type of about twenty nucleotides that are repeated at high number of copies inside each VNTR, and which are [deteceted] detected either by a PCR reaction or a Southern blot hybridization;

4) STSs markers (Simple Tag Sequences), which are unique genomic sequences that can be amplified by a pair of specific oligonucleotide primers and which are generally non polymorphic;

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5) ESTs (Expressed sequence Tags) which are transcribed in mRNS and that can be amplified by a pair of oliqonucleotide primers.

On page 8, replace paragraph 1 beginning at line 1 with the following new paragraph:

The sequences of the DNA markers of the above groups 1) to 4) as well as the sequences of the oligonucleotide detection tools for each of them are [freely] publicly available on electronic databases, particularly on the Internet World Wide Web at the following address: <http://www.ncbi.nlm.nih.gov>.

On page 9, replace the last paragraph beginning at line 19, continuing to page 10, beginning at line 1, with the following new paragraph:

As already mentioned above, the fact that the inventors have now precisely identified the very small chromosomal regions that are subject to frequent genetic alterations allow [the] one <u>of</u> ordinary skill in the art to use any publicly available DNA markers contained in the art that detect a specific chromosomal locus [o interrest] <u>of interest</u> according to the present invention to perform a diagnostic method of the invention or to clone tumor suppressor genes in the corresponding chromosomal regions of [interrest] <u>interest</u>. More particularly, the DNA markers embraced by the present invention cover all the publicly

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available tools spanning these specific chromosomal loci of [interrest] interest, namely, microsatellite DNA markers, namely:

1) Microsatellite DNA markers;

2) RFLP markers (usually constituted by specific oligonucleotide probes);

3) VNTR markers (Variable Number of Tandem Repeats), also named [<<ministallites>>] <<minisatellites>> that are sequences of the <<Alu>> type of about twenty nucleotides that are repeated at high number of copies inside each VNTR, and which are [deteceted] detected either by a PCR reaction or a Southern blot hybridization;

4) STSs markers (Simple Tag Sequences), which are unique genomic sequences that can be amplified by a pair of specific oligonucleotide primers and which are generally nonpolymorphic;

5) ESTs (Expressed Sequence Tags) which are transcribed in mRNA and that can be amplified by a pair of oligonucleotide primers.

On page 10, replace the first paragraph beginning at line 10 with the following new paragraph:

The sequences of the DNA markers of the above groups 1) to 4) as well as the sequences of the oligonucleotide detection tools for each of them are [freely] publicly available on electronic databases, particularly on the Internet World Wide Web at the following address: <http://www.ncbi.nlm.nih.gov>>.

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On page 10, replace the last paragraph beginning at line 14 continuing to page 11, beginning at line 1, with the following new paragraph:

An object of the present invention consists in a composition for the predictive diagnosis of an heptocellular carcinoma in a patient comprising at least a polynucleotide containing a DNA marker which is localized in the following chromosomal regions:

a) 1p;

b) 1q;

c) 2q;

d) 4q;

e) 6p;

f) 7p;

g) 7q;

h) 8p;

i) 8q;

j) 9p;

k) 9q;

l) 10q;

m) 13q;

n) 14q;

o) 16p;

p) 16q;

q) 17p;

r) 17q.

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said DNA markers comprising any of the publicly available markers spanning these specific chromosomal loci of [interrest] interest, namely, mircrosatellite DNA markers, namely:

1) Microsatellite DNA markers;

2) RFLP markers;

3) VNTR markers (Variable Number of Tandem Repeats);

4) STSs markers (Simple Tag Sequences);

5) ESTs (Expressed Sequence Tags).

On page 11, replace the first paragraph beginning at line 8 with the following new paragraph:

Another object of the present invention consists in a composition for the predictive diagnosis of an hepatocellular carcinoma in a patient comprising at least one polynucleotide containing a DNA marker which is localized preferably in the following chromosomal regions:

a) 8p23;

b) 8p122;

c) 8p21;

d) 1p35-p36;

e) 16p23-q24 and

f) 14q32,

said DNA markers comprising any of the publicly available markers spanning these specific chromosomal loci of [interrest] interest, namely, microsatellite DNA markers, namely:

1) Microsatellite DNA markers;

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2) RFLP markers;

3) VNTR markers (Variable Number of Tandem Repeats);

4) STSs markers (Simple Tag Sequences);

5) ESTs (Expressed Sequence Tags).

On page 11, replace the third paragraph beginning at line 28 with the following new paragraph:

A summary of the different loci localization using microsatellite DNA markers in each chromosome pair in human has been described by Dib C. et al. in 1996. The full sequences of the whole [microsatelites] microsatellites DNA markers as well as the full sequences of the amplicons generated using these microsatellite DNA markers are [freely] publicly available on electronic databases (Genbank, STS Bank), more particularly on the Internet World Wide Web at the following address: <http://www.genethon.fr>>

On page 12, replace the first paragraph beginning at line 3 with the following new paragraph:

As already discussed hereinbefore, the DNA fragments that are frequently altered during HCC are strongly thought to carry tumor suppressor genes that are no longer expressed when they are altered in the cancerous tissues. Due to the precision of the new chromosome mapping realized herein by the inventors, it is now possible to achieve the cloning of the wild DNA fragments corresponding to the altered chromosome regions found in HCC tissue samples in order to characterize and sequence the

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candidate tumor suppressor genes carried by these DNA fragments and subsequently expect the use of these cloned genes of interest in the field of [diagnostic] diagnostics and also in the area of therapeutics, specifically for gene therapy.

On page 12, replace the second paragraph beginning at line 12 with the following new paragraph:

Each of the polymorphic microsatellite DNA markers used according to the present invention consists in a pair of specific primers having a sequence that is complementary to a genomic DNA sequence flanking respectively the 5' end and the 3' end of a [higly] highly polymorphic [microstaellite] microsatellite genomic DNA segment constituted by a polymer [Cytidine]Cytosine-Adenine (5'-...CACA...-3') sequence of a known length. A polymorphic microsatellite DNA marker is used to amplify the microsatellite DNA segment which is then identified by its specific length, for example in a polyacrylamide gel electrophoresis in the presence of urea, as described in the Materials and Methods Section.

On page 12, replace the last paragraph beginning at line 34 with the following new paragraph:

[In] Tables 2 and 4 [are represented] represent a summary of microsatellite marker loci undergoing a significant percentage of LOH.

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On page 13, replace the first paragraph beginning at line 1 with the following new paragraph:

The microsatellite DNA markers names used according to the present invention are the scientific conventional names for which, notably, [the] Genethon [oraganism] (Evry, France) has defined specific pairs of primers permitting one to amplify them, each of the said primers being also useful as a specific probe for detecting the corresponding microsatellite DNA marker.

On page 15, replace the second paragraph beginning at line 6 with the following new paragraph:

For the chromosomal 1p35-p36 region (see also Figure 3), the inventors have now detected at least six regions for which is observed a very high percentage of LOH.

On page 15, replace the third paragraph beginning at line 17 with the following new paragraph:

Consequently, the following polymorphic DNA markers D1S2655, D1S199, D1S478, D1S2828, D1S247 and D1S255 are [aslo] also among the preferred markers used in the diagnostic and cloning methods according to the present invention.

On page 15, replace the fifth paragraph beginning at line 22 with the following new paragraph:

The first peak of LOH is seen using the D14S280 polymorphic marker (50% LOH in 10 informative cases). The second peak of LOH is seen using the D14S995 [polymorphioc] polymorphic marker (36%

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LOH in 14 informative cases). The third peak of LOH is seen using the polymorphic marker D14S81 (57% LOH in 28 informative cases). The fourth peak of LOH is seen using the polymorphic marker D14S265 (58% LOH in 12 informative cases). The fifth peak of LOH is seen using the polymorphic marker D14S292 (35% LOH in 17 informative cases).

On page 15, replace the seventh paragraph beginning at line 30 with the following new paragraph:

Consequently, the following polymorphic DNA markers D14S280, D14S995, D14S81, D14S265 and D14S292 are [aslo] <u>also</u> among the preferred markers used in the diagnostic and cloning methods according the present invention.

On page 15, replace the eighth paragraph beginning at line 33 with the following new paragraph:

For the 16q23-q24 chromosomal region (see also Figure 5), the inventors have now detected at least five regions for which is observed a very high percentage of LOH.

On page 15, replace the last paragraph beginning at line 35 continuing to page 16 beginning at line 1 with the following new paragraph:

The first peak of LOH is seen [unsing] <u>using</u> the D16S3098 polymorphic marker (67% LOH in 15 informative cases). The second peak of LOH is seen using the D16S505 polymorphic marker

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On page 16, replace the first paragraph beginning at line 7 with the following new paragraph:

Consequently, the following polymorphic DNA markers D16S3098, D16S505, D16S511, D16S422 and D16S402 are [aslo] also among the preferred markers used in the diagnostic and cloning methods according to the present invention.

On page 16, replace the second paragraph beginning at line 9 with the following new paragraph:

For the 4q35-q36 chromosomal region (see also Fig. 6), the inventors [hace] have now detected several regions for which is observed a very high percentage of LOH.

On page 16, replace the third paragraph beginning at line 11 with the following new paragraph:

The first peak of LOH is seen [unsing] using the D4S400 polymorphic DNA marker (78% LOH in 9 informative cases). A large region has been determined to undergo frequent LOH [occurrences] occurrences, said region being physically comprised between the D4S1572 and the D4S2937 polymorphic DNA marker.

On page 16, replace the fourth paragraph beginning at line 15 with the following new paragraph:

Consequently, the following polymorphic DNA markers D4S400, D4S1572, D41564, D4S2945, D4S1616 and D4S2937 are [aslo] also among the preferred markers used in the diagnostic and cloning methods according to the present invention.

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On page 16, replace the sixth paragraph beginning at line 25 with the following new paragraph:

Preferably, the combination of the polymorphic markers used according to the present invention [choosen] chosen in such a manner that they are selected among the markers for which the highest LOH percentage in HCC has been found by the inventors.

On page 16, replace the seventh paragraph beginning at line 29 with the following new paragraph:

In a preferred embodiment of the combinations of the [microstaellite] microsatellite markers according to the present invention, each combination [comprise] comprises at least one marker for each of the chromosomal regions depicted in Tables 2 and 3.

On page 16, replace the last paragraph beginning at line 32 continuing to page 17 beginning with at line 1 with the following new paragraph:

More preferably, each combination of microsatellite DNA markers [comprise] comprises at least one DNA marker for each chromosomal region depicted in Table 4, thus for each of the following chromosomal regions: 8p, 1p35-p36, 16q23-q24, 4q35-q36 and 14q32. More specifically the combinations preferably comprise at least one marker for each of the following 8p sub-regions: 8p23, 8p22 and 8p21.

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On page 17, replace the second paragraph beginning at line 13 with the following new paragraph:

Thus, another object of the present invention consists in diagnostic methods and diagnostic compositions using or comprising the above described combinations of [microstallite] microsatellite polymorphic DNA markers.

On page 17, replace the third paragraph beginning at line 14 with the following new paragraph:

In one preferred embodiment of the diagnostic compositions comprising the above described combinations of DNA markers, a single DNA marker is [choosen] chosen in each group.

On page 17, replace the fifth paragraph beginning at line 20 with the following new paragraph:

The results described herein have allowed the inventors to discover that correlations occur between specific alterations in different chromosomal loci amplified with the polymorphic DNA markers used according to the present invention incase of HCC. More precisely, the inventors have observed that the frequency of LOH identified concomitantly on both arms 1p and 13q, 1p and 8p, as well as 6q and 13q [are] is significantly higher in tumors arising from chronic hepatitis lesions (CH) than liver cirrhosis (LC), the numbers of HCCs with CH vs. LC showing LOH in the above combinations being [15 vs.5] 15 vs. 5, 16 vs. 6, and 14 vs. 3 respectively.

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On page 17, replace the sixth paragraph beginning at line 28 with the following new paragraph:

Consequently, diagnostic compositions comprising a specific combination of markers for which a correlation of LOH [have] has been determined are useful in order to help the practitioner to discriminate between HCCs with liver cirrhosis and HCCs with chronic hepatitis lesions.

On page 18, replace the last paragraph beginning at line 27 continuing to page 19 line 1 with the following new paragraph:

Thus, [are] also part of the present invention are diagnostic compositions comprising a combination of microsatellite DNA markers, each combination containing at least one DNA marker [choosen] chosen [in every] from one of the following groups:

a) [Microstallite] Microsatellite markers of 16p, [choosen] chosen among D16S521, D16S407, D16S420 and D16S411;

b) Microsatellite markers of 17p, [choosen] chosen among D17S933, D17S787, D17S949, D17S784 and D17S928,

it being understood that the occurrence of LOH using the above diagnostic compositions are useful to diagnose invasive tumors and that the absence of LOH using these diagnostic compositions will mean a strong support for either early tumors diagnosis or the absence of an HCC.

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On page 20, replace the first paragraph beginning at line 4 with the following new paragraph:

The discovery of the inventors that specific small chromosomal regions are now mapped for frequent genetic alteration during an HCC is allowing [the] one <u>of</u> skill in the art to identify and clone the tumor suppressor genes that have been altered in [case] <u>cases</u> of an HCC.

On page 20, replace the second paragraph beginning at line 7 with the following new paragraph:

The inventors have now precisely mapped the chromosomal regions undergoing frequent genetic alterations, specifically allelic imbalance, the distance between the different DNA markers used being from 2 centimorgans (cM) for the markers the most distant one from each other and being less than 0.25 cM for the markers that are the less distant one form each other, it being generally accepted that 1 cM represents approximately 1000 kilobases +/- 20%. Thus, for the nearest markers, specifically in the 8p region, they are distant on the genome of less than 0.25 cM, or in other words they are distant of less sthan 250 kilobases, and sometimes less than about 100 kb, [specially] <u>especially</u> for the microsatellite markers localized in 8p21, 8p22 and 8p23.

On page 20, replace the third paragraph beginning at line 16 with the following new paragraph:

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In order to isolate candidate tumor suppressor genes localized between the chromosomal positions of two DNA markers according to the present invention, [it is first proceeded with the isolation of] isolate at least one yeast artificial chromosome (YAC) clone which are known to span the genomic DNA between the loci of interest. The YAC libraries are publicly available in Genethon (Evry, France). For the 8p region, the following YACs are used: 852d10 (spanning a chromosomal region containing at least from D8S518 to AFM249WA9 [microstallite] microsatellite DNA markers localizations), 787c11 (from D8S265 to WI-9756), 842b11 (From D8S518 to AFM249WA9), 920h7 (from D8S518 to AFM249WA9), 764c7 (from WI-3823 to D8S1706), 792a6 (from D8S277 to WI-8327), 879f11 (from D8S561 to WI-8327), 910d3 (from D8S561 to D8S1819), 910f12 (from D8S561 to WI-3823), 967c11 (from D8S277 to WI-8327), 918c6 (from D8S561 to D8S1819) and 856d8 (from D8S561 to D8S1819).

On page 21, replace the second paragraph beginning at line 5 with the following new paragraph:

Cosmid libraries are constructed from the YAC clone spanning the 8p23 genomic DNA. For example, the cosmid library is constructed following the technique described by Shimizu et al. in 1990. Briefly, the cosmid vector pWEX15 (Wahl et al., 1987), whose unique BamHI site is filled by Klenow enzyme and converted into a unique XhoI site using an oligonucleotide linker (5'CCTCGCGAGG-3'). pWEX15 is then digested with XhoI and partially filled in with dCTP and dTTp by [klenow] Klenow enzyme,

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leaving 5'-TC-3' at the 5-end. Genomic DNA isolated from the YAC clone of interest is partially digested with a suitable restriction enzyme, for example Sau3AI and fractioned by sucrose-density gradient centrifugation to yield small DNA fragments (30-100 kb). Fragmented DNA is then partially filled in with dATP and dGTP by Klenow enzyme, leaving 5'-GA-3' at the 5'end. Ligation is performed using 0.5μg of vector and from 1 to 2 μg, preferably 1.2 μg, of genomic DNA by a standard method and packaged with in vitro packaging extracts (Gigapack Gold, commercialized by Strategene).

On page 21, replace the fourth paragraph beginning at line 27 with the following new paragraph:

In order to construct a contig map of the above selected cosmids, five ng of each cosmid DNA is digested with a restriction endonuclease, preferably EcoRI, electrophoresed on 1.0% agarose gels, and subjected to Southern blotting, using each cosmid as a probe. To suppress background signals generated by repetitive sequences present in cosmid inserts, an excess of total human DNA is prehybridized with each radiolabelled probe before [hybridisation] <u>hybridization</u> begins. The contig map of these cosmid clones is constructed on the basis of the hybridization patterns.

On page 21, replace the last paragraph beginning at line 34 continuing to page 22 beginning at line 1 with the following new paragraph:

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Then, exon amplification is performed as described by Buckler et al. (1991), the Materials and Methods section of this article being herein incorporated by reference. Briefly, fragments of cosmid DNAs are subcloned into a plasmid vector, pSPL1, and transfected into COS-7cells by electroporation. Reverse transcriptase (RT)-PCR products are isolated from cytoplasmic RNA of their transfectants and confirmed by Southern [Hybridisation] Hybridization to have originated from the appropriate cosmid clones.

On page 22, replace the second paragraph beginning at line 10 with the following new paragraph:

Then, Northern blot analyses are performed using Multitissue blots obtained from Clontech labs (Palo Alto, CA). Prehybridization[,] and hybridization are performed according to the manufacturer's recommendation in a solution containing 50% formamide, 5 x Denhardt's solution, 6 x SSC and 1% salmon sperm DNA. A restriction endonuclease (for example EcoRI/XhoI) cleavage product of the cDNA insert is used as a probe. Filters are then washed in 0.1 x SSC/0.1% SDS at 50°C for 29 min twice.

On page 24, replace the second paragraph beginning at line 7 with the following new paragraph:

[Are also] Also part of the present invention are the amplified nucleic fragments (<<amplicons>>) defined herein above.

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On page 24, replace the seventh paragraph beginning at line 28 continuing to page 25 beginning at line 1 with the following new paragraph:

The SDA technique was initially realized [unsing] using the restriction endonuclease HincII but is now generally [pratised] practiced with an endonuclease from *Bacillus stearothermophilus* (*BSO*BI) and a fragment of a DNA polymerase which is devoid of any 5'→3' exonulcease activity isolated from *Bacillus cladotenax* (exo-*Bca*) [=exo-minus-*Bca*]. Both enzymes are able to operate at 60°C and the system is now optimized in order to allow the use of dUTP and the decontamination by UDG. When [unsing] using this technique, as described by Spargo et al. in 1996, the doubling time of the target DNA is of 26 seconds and the [amplification] amplification rate is of 1010 after an incubation time of 15 min at 60°C.

On page 25, replace the second paragraph beginning at line 6 with the following new paragraph:

Thus, another object of the present invention consists in using the nucleic acid fragments according to the invention (primers) in a method of DNA or RNA amplification according to the SDA technique. For performing of SDA, two pairs of primers are used: [apair] a pair of external primers (B1, B2) consisting in a sequence specific of the target polynucleotide of interest and a pair of internal primers (S1, S2) consisting in a fusion

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oligonucleotide carrying a site that is recognized by a restriction endonuclease, for [exemple] example the enzyme *BSO*BI.

On page 25, replace the fourth paragraph beginning at line 24 with the following new paragraph:

More specifically, the following conditions are used when performing the SDA amplification reaction with the primers of the invention designed to contain a BsoBI restriction site: *Bso*BU.exi *Bca* [=exo-minus-*Bca*] SDA reactions are performed in a 50μl volume with final concentrations of 9.5 mM MgCl2, 1.4 mM each dGTP, DATP, TTP, dCTP-alpha-S, 100 μg/ml acetylated bovine serum albumin, 10 ng/ml human placental DNA, 35 mM K2HPO4 pH 7.6, 0.5 μM primers S1 BsoBI and B2 BsoBI, 0.05 μM primers B1 BsoBI and B2 BsoBI, 3.2 U/μl BsoBI enzyme, 0.16 U/μl exo Bca [=exo-minus-Bca] enzyme, 3mM Tris-HCl, 11mM NaCl, 0.3 mM DTT, 4 mM KCl, 4% glycerol, 0.008mM EDTA, and varying amounts of target DNA. Prior to the addition of BsoBI and exo Bca, [icomplete] incomplete reactions (35 μl) are heated at 95°C for 3 min to denature the target DNA, followed by 3 min at 60°C to anneal the primers. Following the addition of a 15 μl enzyme mix consisting of 4 μl of BsoBI (40 Units/μl), 0.36 μl exo BCA (22 Units/μl), and 10.6 μl enzyme dilution buffer (10 mM Tris Hcl, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT), the reactions are incubated at 60°C for 15 min. Amplification is terminated by heating for 5 min in a boiling water bath. A no-SDA sample is created by heating a sample in a boiling water bath. A no-SDA sample is created by heating a

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sample in a boiling water bath immediately after enzyme addition. Aerosol resistant tips from Continental Laboratory Products are used to reduce contamination of SDA reactions with previously amplified products.

Page 27, replace the first paragraph beginning at line 7 with the following new paragraph:

The oligonucleotide probes according to the present [ivention] invention hybridize specifically with a DNA or RNA molecule comprising all or part of one polynucleotide among the selected candidate tumor suppressor gene under stringent conditions.

Page 27, replace the last paragraph beginning at line 27 continuing to page 28 beginning with line 1 with the following new paragraph:

In the latter case, other labeling techniques may be also used such as those described in the [french] French patents FR-2,422,956 and 2,518,755. The hybridization step may be performed in different ways (Matthews et al., 1988). The more general method [concists] consists in immobilizing the nucleic acid that has been extracted from the biological sample on a substrate (nitrocellulose, nylon, [polystyren] polystyrene) and then to incubate, in defined conditions, the target nucleic acid with the probe. [Subsequently] Subsequent to the hybridization step, the excess amount of the specific probe is discarded and the hybrid

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molecules formed are detected by an appropriate method (radioactivity, fluorescence or enzyme activity measurement).

On page 28, replace the second paragraph beginning at line 7 with the following new paragraph:

In another advantageous embodiment of the probes according to the present invention, the [latters] latter may be used as <<capture probes>>, and are for this purpose immobilized on a substrate in order to capture the [targer] target nucleic acid contained in a biological sample. The captured target nucleic acid is subsequently detected with a second probe which recognizes a sequence of the target nucleic acid which is different from the sequence recognized by the capture probe.

On page 28, replace the third paragraph beginning at line 13 with the following new paragraph:

The oligonucleotide fragments useful as probes or primers according to the present invention may be prepared by cleavage of the polynucleotides of the selected candidate tumor suppressor gene by restriction enzymes, [the] one of skill in the art being guided by the procedures described in Sambrook et al. in 1989.

On page 28, replace the fifth paragraph beginning at line 25 with the following new paragraph:

A chemical method for producing the nucleic acids according the invention which have a length of more than 200 nucleotides

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nucleotides (or 200 bp if these molecules are double stranded) comprises the [fllowing] following steps:

- assembling the chemically [synthsised] synthesized oligonucleotides, having different restriction sites at each end.
- cloning the thus obtained nucleic acids in an appropriate vector.
- purifying the nucleic acid by hybridizing an appropriate probe according to the present invention.

On page 28, replace the last paragraph beginning at line 33 continuing to page 29 the first line with the following new paragraph:

In the case in which the above nucleic acids are used as coding sequences in order to produce a polypeptide according to the present invention, it is important to ensure that their sequences are compatible (in the appropriate reading frame) with the [aminoacid] amino acid sequence of the polypeptide to be produced.

On page 29, replace the first paragraph beginning at line 3 with the following new paragraph:

The oligonucleotide probes according to the present [ivention] invention may also be used in a detection device comprising a matrix library of probes immobilized on a substrate, the sequence of each probe of a given length being localized in a shift of one or several bases, one from the other, each probe of the matrix library thus being complementary of a distinct

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sequence of the target nucleic acid. Optionally, the substrate of the matrix may be a material able to act as an electron [donnor] donor, the detection of the matrix [poisitons] positions in which an hybridization has occurred [beeing] being subsequently determined by an electronic device. Such matrix libraries of probes and methods of specific detection of a [targer] target nucleic acid [is] are described in the European patent application No US-5,202,231 (Drmanac).

On page 29, replace the third paragraph beginning at line 14 with the following new paragraph:

An oligonucleotide probe matrix may [advantadgeously] advantageously be used to detect mutations occurring in the selected candidate tumor suppressor gene. For this particular purpose, probes are specifically designed to have a [nucleotidic] nucleotide sequence allowing their hybridization to the genes that carry known mutations (either by deletion, insertion of substitution of one or several nucleotides). By known mutations is meant mutations on [the] the selected candidate tumor suppressor gene that have been identified.

On page 29, replace the third paragraph beginning at line 21 continuing to page 30 with the following new paragraph:

Another technique that is used to detect mutations in the selected candidate tumor suppressor gene is the use of a high-density DNA array. Each oligonucleotide probe constituting a unit element of the high density DNA array is designed to match a

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specific subsequence of [the] the selected candidate tumor suppressor gene genomic DNA or cDNA. Thus, an array consisting of oligonucleotides complementary to subsequences of the target gene sequence issued to determine the identity of the target sequence with the wild gene sequence, measure its amount, and detect differences between the target sequence and the reference wild gene sequence of [the] the selected candidate tumor suppressor gene. In one such design, termed 4L tiled array, is implemented a set of four probes (A, C, G, T), preferably 15-nucleotide oligomers. In each set of four probes, the perfect complement will hybridize more strongly than mismatched probes. Consequently, a nucleic acid target of length L is scanned for mutations with a tiled array containing 4L probes, the whole probe set containing all the possible mutations in the known wild [refrence] reference sequence. The hybridization signals of the 15-mer probe set tiled array are perturbed by a single base change in the target sequence. As a consequence, there is a [caharacteristic] characteristic loss of signal or a <<footprint>> for the probes flanking a mutation position. This technique was described by Chee et al. in 1996.

On page 30, replace paragraph 3 beginning at line 15 with the following new paragraph:

The present invention also pertains to a family recombinant plasmids [chaarcterized] characterized in that they contain at least a nucleic acid according the above teachings. According to an advantageous embodiment, a recombinant plasmid comprises a

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polynucleotide of the selected candidate tumor suppressor gene, or one nucleic fragment thereof.[.]

On page 30, replace paragraph 4 beginning at line 20 with the following new paragraph:

Another object of the present invention consists in an appropriate vector for cloning, expressing or inserting a nucleic acid sequence, characterized in that it comprises a nucleic acid as above described in a site nonessential for its replication, optionally under the control of the regulation elements allowing the expression of a polypeptide of the invention.

On page 31, replace the first paragraph beginning at line 4 with the following new paragraph:

The step d) of the above-described method may consist in a Single-[Starnd] Strand Polymorphism technique (SSCP), a Denaturing Gradient Gel Electrophoresis (DGGE), or the FAMA technique described in the PCT patent application No Wo-95/07361.

On page 31, replace the second paragraph beginning at line 7 with the following new paragraph:

Another object of the present invention consists in a method for detecting a genetic abnormality linked to the HCC in a biological sample containing DNA or cDNA, comprising the steps of:

a) bringing the biological sample into contact with an oligonucleotide probe according to the invention, the DNA

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contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the biological sample;

b) detecting the hybrid formed between the oligonucleotide probe and the DNA [conatained] contained in the biological sample.

On page 31, replace the third paragraph beginning at line 16 with the following new paragraph:

The present invention consists also in a method for detecting a genetic abnormality linked to the HCC in a biological sample containing DNA, comprising the steps of:

a) bringing into contact a first oligonucleotide probe according to the invention that has been immobilized on a [suuport] support, the DNA contained in the sample having been optionally made available to hybridization and under conditions permitting a hybridization of the primers with the DNA contained in the biological sample;

b) bringing into contact the hybrid formed between the immobilized first oligonucleotide probe and the DNA contained in the biological sample with a second oligonucleotide probe according to the invention, which second probe hybridizes with a sequence different from the sequence to which the immobilized first probe hybridizes, optionally after having removed the DNA contained in the biological sample which has not hybridized with the immobilized first oligonucleotide probe.

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On page 31, replace the last paragraph beginning at line 30 with the following new paragraph:

Another object of the present invention consists in a method for detecting a genetic abnormality linked to the HCC in a biological sample containing DNA, by the detection of the presence and of the position of base substitutions or base deletions in a nucleotide sequence included in a double stranded DNA preparation to be tested, the said method comprising the steps of:

a) amplifying specifically the region containing, on one hand, the nucleotide sequence of the DNA to be tested and on the other hand the nucleotide sequence of a DNA of known sequence, the DNA of known sequence being a polynucleotide according to the invention;

b) labeling the sense and antisense strands of these DNA with [diferent] <u>different</u> fluorescent or other non-isotopic labels;

c) hybridizing the amplified DNAs;

d) revealing the heteroduplex formed between the DNA of known sequence and the DNA to be tested by cleavage of the mismatched parts of the DNA strands.

Such a mismatch localization technique has been described by Meo et al. in the PCT application No WO-95/07361.

On page 32, replace the third paragraph beginning at line 24 with the following new paragraph:

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SSCP analysis is performed by PCR amplification [o] of each of the determined exons of the identified candidate tumor suppressor gene, [correspoinding] corresponding to the coding region, after having designed the suitable specific oligonucleotide primers, for example following the teachings of Rolfs et al. (1992). For this particular purpose, PCR reactions are carried out in 5-μl solutions containing 100 ng genomic DNA, 1 μM each primer, 25 μM dNTP, 2 μCi of [a32P]dCTP (Amersham), and 0.25 U of Taq1 polymerase (Boehringer Mannheim). PCR products, which [sow] show variant bands by SSCP analysis, are cloned into HindIII site of pBluescript SK(-) and the resulting independent clones are polled. Both strands are sequenced by the [dideoxu] dideoxy chain-termination method with T7 DNA polymerase.

On page 33, replace the first paragraph beginning at line 3 with the following new paragraph:

Other techniques for detecting the occurrence of a genetic alteration (insertions, deletions, substitutions) either generally in the chromosomal loci of [interrest] interest identified by the inventors or in the specific sequences of the candidate suppressor genes determined as described herein before.

Page 33, replace the second paragraph beginning at line 7 with the following new paragraph:

By performing a band shift assay, if the mutation in the selected candidate tumor suppressor gene is a deletion or insertion of one or more bases, a small segment of the gene-

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including the site of the mutation- is amplified by PCR, and the mutated allele detected by gel electrophoresis because of its altered mobility. Separation of 1 bp differences requires the incorporation of a radioactive label into the PCR product, followed by electrophoresis on a large denaturing polyacrylamide gel and [autoradioragraphy] autoradiography. Differences of two or more bases are resolved on non-denaturing polyacrylamide gels, and the DNA fragments detected by stained with ethidium bromide (Sambrook et al., 1989).

On page 33, replace the fourth paragraph beginning at line 23 with the following new paragraph:

A third suitable procedure for detecting mutation in the selected candidate tumor suppressor gene consists in an allele-specific oligonucleotide assay, a technique in which the PCR product is spotted onto a nylon or [notro cellulose] nitrocellulose membrane which is then incubated with a radioactively labeled oligonucleotide sequence of about 18 bases corresponding to either the normal or the mutant sequence (Conner et al., 1983; Saiki et al., 1986; Saiki et al., 1989). The short oligonucleotide probes bind to their exact complementary sequence, provided that the [tempreature] temperature and salt concentration of the solution used for the incubation are carefully controlled (see Rolfs et al., 1992 for the reagents concentrations determination). The oligonucleotide probe [cans] can also be labeled with a non-radioactive tag such as biotin (saiki et al., 1986).

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On page 33, replace the last paragraph beginning at line 32 continuing to page 34 with the following new paragraph:

The allele-specific priming technique is also very useful in order to detect genetic alterations in the selected candidate tumor suppressor gene. This technique utilizes the specificity of the PCR priming process to effect allele-specific priming of normal or mutant sequences (Newton et al., 1989; Ferrie et al., 1992). The allele-specific primer is so designed that its 3'end is located exactly at the site of mutation. PCR amplification occurs between this primer and a <<common primer>>, some distance away on the other side of the mutation, only if the sequence at the 3'end of the [alle] allele-specific primer matches the sequence of the sample DNA at this point.

On page 34, replace the third paragraph beginning at line 15 with the following new paragraph:

The heteroduplex analysis method is based upon the observation that a hybrid between two single-stranded DNA molecules with sequences which differ from each other by single nucleotide has an altered conformation, which is detected as a reduction in electrophoretic mobility on non-denaturing gel. Briefly, the formation of heteroduplexes after PCR is encouraged by a [bried] brief denaturation step, followed by slow cooling at room temperature. The DNA is then electrophoresed in a non-denaturing gel of either polyacrylamide or Hydrolink (AT Biochem), and stained with ethidium bromide. The technical

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details regarding the specific procedures employed are described by Keen et la. (1991), Whiteet al. (1992) and Soto et al. (1992).

On page 34, replace the fourth paragraph beginning at line 24 with the following new paragraph:

The mutations occurring in the selected candidate tumor suppressor gene are also detected by the Denaturing gel electrophoresis (DGGE), a technique that exploits the fact that if a DNA fragment is electrophoresed at high temperature in a polyacrylamide gel which contains increasing concentrations of denaturants, it will become partially or completely denatured at some point. This event [produices] produces a sharp reduction in its electrophoretic mobility. Preferably, the sensitivity of the method is increased by the attachment of a GC-rich sequence (<<GC-clamp>>) to the end of the DNA fragment during PCR, which then serves as the last melting domain. Mutations of up to 600 bp are rapidly detected using the DGGE method. The specific procedures are [descrobed] described by Grompe (1993), Fischer et al. (1983), Shieffield et al. (1989).

On page 34, replace the last paragraph beginning at line 34 continuing to page 35 with the following new paragraph:

[Are also] Also used are the Chemical cleavage method (CCM) and a very useful improvement of such a method which is FAMA. CCM is based upon the susceptibility of mismatched bases in a heteroduplex to modification by chemicals. DNA from the test sample and a radioactively labeled control is mixed, denatured,

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and allowed to form a heteroduplex. Incubation with hydroxylamine or osmium tetroxide results in modification of mismatched cytosines or thymines respectively, which are then cleaved with piperidine. The cleavage product produced by the mismatch is then detected by electrophoresis and autoradiography (Cotton et al., 1988; Montandon et al., 1989; Saleeba et al., 1992; Haris et al., 1994).

On page 35, replace the first paragraph beginning at line 8 with the following new paragraph:

It is now easy to produce proteins in high amounts by the genetic engineering techniques by the use, as expression vectors, plasmids, phages or phagemids. The polynucleotides that code for the polypeptides of the present invention [is] are inserted in an appropriate expression vector in order to produce *in vitro* [produce] the polypeptide of interest.

On page 35, replace the second paragraph beginning at line 12 with the following new paragraph:

Thus, the present invention also concerns a method for the producing a polypeptide encoded by a candidate tumor suppressor gene of the invention, [the] said method comprising the steps of:

a) Optionally amplifying the nucleic acid coding for the desired polypeptide using a pair of primers according to the invention (by SDA, TAS, 3SR NASBA, TMA etc.).

b) Inserting the nucleic acid of interest in an appropriate vector;

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c) culturing, in an appropriate culture medium, a cell host previously transformed or transfected with the recombinant vector of step b);

d) harvesting the culture medium thus conditioned or [lyse] lysing the cell host, for example by sonication or by an osmotic shock;

e) separating or purifying, from the said culture medium, or form the pellet of the resultant host cell lysate the thus produced polypeptide of interest.

f) Characterizing the produced polypeptide of interest.

On page 35, replace the third paragraph beginning at line 25 with the following new paragraph:

The polypeptides encoded to the candidate tumor suppressor genes according to the invention may be characterized by binding onto an immunoaffinity chromatography column on which polyclonal or monoclonal [antiibodies] antibodies directed to a polypeptide among the polypeptides of the selected candidate tumor suppressor gene have previously been immobilized, before their sequencing [unsing] using the conventional protein sequencing methods well known from [the] one of skill in the art.

On page 35, replace the last paragraph beginning at line 31 continuing to page 36 with the following new paragraph:

The said antibodies may be prepared from hybridomas according to the technique described by Kohler and Milstein in

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1975. The polyclonal antibodies may be prepared by [immunisation] immunization of a mammal, especially a mouse or a rabbit, with a peptide according to the invention that is combined with an adjuvant of immunity, and then by purifying the specific antibodies contained in the serum of the immunized animal onto an affinity chromatography column on which has previously been immobilized the peptide that has been used as the antigen. A technique for preparing and using a immunoaffinity chromatography column is described, for example, by Bird et al. in 1984.

On page 36, replace the first paragraph beginning at line 5 with the following new paragraph:

A [prefered] preferred embodiment for preparing antibodies raised against the candidate tumor suppressor gene encoded protein is described hereafter. Briefly, the polypeptide of [interrest] interest is conjugated to egg albumin (Calbiochem) using the benzidine-bis-diazoted procedure described by Gregory et al. in 1967, the ratio of polypeptide residues to one molecule of ovalbumin being 5:1. Rabbits are injected at time 0 with 1 mg of the conjugated polypeptide. Two months after the primary injection, animals are injected with 0.5 mg of the conjugated polypeptide and a [thirs] third injection of 0.5 mg of the same polypeptide is performed between two and four months after the [seecond] second injection. Antiserum is harvested between two and four weeks following the third conjugated polypeptide injection and optionally purified onto an affinity chromatography

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column is previously described. Preferably the injection is an intradermally multi-points injection; generally ten points of injection are performed.

On page 36, replace the last paragraph beginning at line 33 continuing to page 37 with the following new paragraph:

The choice of a determined promoter, among the above-described promoters is well in the ability of one of skill in the art, guided by his knowledge in the genetic engineering technical field, and by being also guided by the gook of Sambrook et al. in 1989 or also by the procedures described by Fuller et al. in 1996.

On page 37, replace the third paragraph beginning at line 11 with the following new paragraph:

Other suitable vectors for the expression of the protein encoded by a candidate tumor suppressor gene above-defined or their peptide fragments in a baculovirus expression system consist in plasmids which are baculovirus expression vectors with multiple cloning sites (MCS) that contain the specific expression elements of the pol gene in a pUC8 backbone. These plasmids can be divided into two subgroups, namely, on one hand the vectors pVLMelMyc-, which allow the construction of a N-terminal [fucion] fusion to the signal sequence of the melittin gene (Chai et al., 1993; Vlasak et al., 1983) and on the other hand the vectors pVLPolMyc- which allow a N-terminal fusion to the first 12 aa of the pol and the c-Myc tag. The gene to be expressed can be

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cloned into the MCS, resulting in an N-terminal fusion to either the mel-myc or th epol-myc which are encoded by the vectors. An example of using such versatile vectors to express a mouse heterologous protein (5HT5A serontonin receptor) is notably described by Lenhardt et al. in 1996.

On page 37, replace the fourth paragraph beginning at line 24 with the following new paragraph:

Another suitable vector for performing the above-described process is a vaccinia virus [vactor] vector. In this specific embodiment, BSC-40 or LoVo are used for the transfection and culture steps.

On page 38, replace the third paragraph beginning at line 10 with the following new paragraph:

The purification of the recombinant protein, peptide or oligomeric peptide according to the present invention may be realized by passage onto a Nickel or [Cupper] Copper affinity chromatography column. The Nickel chromatography column may contain the Ni-NTA resin (Porath et al., 1975).

On page 42, replace the first paragraph beginning at line 1 with the following new paragraph:

We were then interest in exploring a possible correlation between clinicopathological characteristics of the tumors and LOH. Because the limited number of samples showing LOH at each individual locus and for which clinicopathological parameters

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were available could not confer any significant statistical value, we performed the analysis at the level of each chromosomal arm. The data are summarized in Table 3. Noen of the chromosome arm alteration was statistically correlated with positive serum markers (HBsAG or HCVAb) for hepatitis virus infections. Although the relationship between LOH and the tumor stage could not be statistically evaluated because of the low number of early tumors, a tendency towards frequent LOH on 1p and 1q was observed in small HCCs classified as T1 (respectively 4 and 5 of 10 tumors). On the contrary, at this tumor stage, few changes were noted on 2q, 6q, 7q, 8q, 14q, 16pq and 17pq (0-1 of 10 tumors). Allelic imbalance on 16p and 17p appeared relatively frequently in invasive tumors having intraheptatic metastasis or portal vein invasions compared to non-invasive tumors (3-4/12 vs. 1/13 tumors). Pathological informations of the adjacent non-tumorous liver counterparts were obtained from 66 cases, 35 of which displayed chronic hepatitis (CH) lesions and the remainder (31) liver cirrhosis (LC). No statistically [significative] <u>significant</u> correlation was observed between the presence of genetic alterations on a particular chromosomal arm and the pathological state of the non tumorous liver. However, the frequency of LOH observed concomitantly on both arms 1p and 13q, 1p and 8p as well as 6q and 13q [were] <u>was</u> significantly higher in tumors arising from CH than LC (the number of HCCs with CH vs. LC showing LOH in above combinations were 16 vs. 5, 16 vs. 6, and 14 vs. 3 respectively).

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Amendments to the Claims

Please amend claim 18 as follows:

18. (Amended) A tumor suppressor gene polynucleotide involved in the occurrence of a HCC in a patient obtained [according to the method of claim 17] by a process comprising:

a) constructing a cosmid library from a selected YAC clone;

b) selecting cosmid clones of interest by colony hybridization with labeled human genomic DNA as a probe;

c) constructing a contig map of the purified selected cosmid clones;

d) performing an exon amplification reaction and inserting the reverse transcribed RNA fragments in a suitable human cDNA library, preferably a fetal or adult liver cDNA library and selecting the hybridizable cDNA clones; and

f) sequencing the selected cDNA clones inserts and characterizing the coding sequences.

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